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REGULAR ARTICLE

Effects of including forbs on N_2-fixation and N yield in red clover-ryegrass mixtures

Nawa Raj Dhamala · Jim Rasmussen · Georg Carlsson · Karen Søegaard · Jørgen Eriksen

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Abstract
Background Legume biological nitrogen (N_2)-fixation is stimulated by neighbouring non-fixing species, but studies of legume N_2-fixation in temporary grasslands including non-leguminous forage herb (forb) species are rare.
Methodology We investigated N_2-fixation, N yield, and total herbage production in a range of species mixtures consisting of three forb species—chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.), and caraway (*Carum carvi* L.)—mixed into a traditional red clover (*Trifolium pratense* L.) and perennial ryegrass (*Lolium perenne* L.) mixture at two fertilisation levels.
Results The percentage of red clover N derived from the atmosphere (%Ndfa) was higher in mixtures containing non-legumes than in pure stand but, did not increase with inclusion of forbs. On a whole-seasonal basis, red clover in mixtures derived 90% or more of its N from fixation even when fertilised with 216 kg total N ha^{-1}. Forbs, in particular chicory, reduced the amount of N_2-fixation and total N yields by affecting the red clover proportion in the harvested biomass.
Conclusions Generally, inclusion of forbs in red clover-ryegrass mixtures had no negative effect on total herbage production and percentage of legume N_2-fixation. However, to maintain a high total N and N_2-fixation yields, mixtures should not include a high seeding proportion of chicory.

Keywords Plant diversity · Temporary grassland · Chicory · Ribwort plantain · Caraway · Percentage of N_2-fixation (%Ndfa)

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N. R. Dhamala (✉) · J. Rasmussen · K. Søegaard · J. Eriksen
Department of Agroecology, Aarhus University, PO Box 50, 8830 Tjele, Denmark
e-mail: nawadhamala@gmail.com

G. Carlsson
Department of Biosystems and Technology, Swedish University of Agricultural Sciences, PO Box 103, 23053 Alnarp, Sweden

Introduction

The inclusion of forage legumes in grassland production systems has been shown to improve forage quality (Lüscher et al. 2014), enhance soil N availability (Fustec et al. 2010), and increase plant productivity (Nyfeler et al. 2011). Soil N availability is enhanced through the process of biological N_2-fixation (BNF), N rhizodeposition (Høgh-Jensen and Schjoerring 2001; Rasmussen et al. 2007), and turnover and senescence of above- and below-ground plant residues (Rasmussen et al. 2008; Dahlin and Stenberg 2010b; Rasmussen et al. 2012). The benefits of forage legumes also includes their green manuring and catch crop capacities in temporary grasslands included in rotation, where the buildup of the soil N pool is mineralised upon termination of the swards, rendering it available to the subsequent crop (Eriksen et al. 2008).

Globally, forage legumes are the second largest source of BNF after grain legumes (Herridge et al. 2008). However, there are considerable spatial and temporal variations in their abilities of BNF (Lüscher et al. 2014; Anglade et al. 2015). The extent of legume BNF is influenced by several internal and external factors such as legume plant and rhizobium genotype and their interaction with the surrounding environment, including management practices such as cutting, grazing, fertilisation, and species composition including functional diversity of legumes and non-legumes (Carlsson and Huss-Danell 2003).

Numerous studies have shown that plant diversification by growing forage legumes in mixtures with non-legumes, notably forage grasses, has a direct effect on the extent to which legumes rely on BNF for their N acquisition (e.g. Høgh-Jensen and Schjoerring 1997; Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2009). The inclusion of a non-legume increases the competition for available soil N and increases legume dependence on BNF compared to legumes grown in pure stands (Høgh-Jensen and Schjoerring 1997; Carlsson and Huss-Danell 2003; Hauggaard-Nielsen et al. 2009). Therefore, many studies have focused on legume and non-legume diversity in grasslands to improve BNF and soil N availability. However, the majority of studies on N dynamics in grasslands tend to be confined to binary mixtures of grass and clover. Studies on BNF in multi-species grasslands including non-leguminous forage herbs (forbs) are scarce (exceptions: Pirhofer-Walzl et al. 2012; Frankow-Lindberg and Dahlin 2013).

Chicory (*Cichorium intybus* L.), ribwort plantain (*Plantago lanceolata* L.), hereafter plantain, and caraway (*Carum carvi* L.) are three forbs that may be adopted in grasslands. They have potential as important components of grasslands due to their high competitive abilities in mixtures with different forage legume and non-legume species (Søegaard et al. 2013). They increase plant diversity and herbage production (Søegaard et al. 2011; Cong et al. 2017), forage quality (Høgh-Jensen et al. 2006; Søegaard et al. 2008), and mineral nutrition (Pirhofer-Walzl et al. 2011) and can tolerate adverse weather conditions (Younie 2012). They have deep and diverse root systems (Stewart 1996; Li and Kemp 2005) and can take up N from deeper soil layers (Thorup-Kristensen 2006; Pirhofer-Walzl et al. 2013). Thus, the synergistic effects of including forbs with different above- and below-ground traits in grass-clover mixtures are expected to increase soil N acquisition and the competition for available soil N with accompanying legume species, thereby affecting the dependence of the legume species on BNF. However, forbs are not widely included in grasslands in Europe, and little is known about how different forbs in grass-clover mixtures influence legume BNF. Here, we conducted an experiment with the objectives of determining how the inclusion of non-leguminous forb species in mixtures of red clover (*Trifolium pratense* L.) and perennial ryegrass (*Lolium perenne* L.) would affect sward composition (red clover, ryegrass, and forbs) in terms of dry matter (DM) production and N accumulation as well as the percentage (%Ndfa) and amounts of red clover BNF when exposed to two levels of fertilisation. The following hypotheses were tested: (1) percentage of red clover N derived from the BNF (%Ndfa) can be increased by increasing plant species diversity via the inclusion of companion non-legume forbs due to functional complementarity between the species and (2) cattle slurry application will reduce the red clover dependency on BNF.

Materials and methods

Experimental site

The field experiment was carried out at Foulumgaard Experimental Station, Aarhus University, in Central Jutland, Denmark (56° 29° N and 09° 34° E). The experimental field was part of an organic dairy crop rotation with a cropping history of temporary grasslands and arable crops since 1987. The soil is a loamy sand characterised as Typic Hapludult with 7.7% clay and 1.6% carbon (Eriksen et al. 2015) and 0.14% total N. Extractable P was 22 mg kg^{-1}, exchangeable K was 64 mg kg^{-1} and pH was 5.9. The mean monthly temperatures during the experimental period (April–October, 2014) were between 8 and 19 °C, with July the warmest months. The monthly precipitation varied between 35 and 117 mm, with May, August, and October being relatively damp (Fig. 1). The average temperature for the experimental period was 13.7 °C and the average monthly precipitation was 65 mm. The 30-year average of temperature and monthly precipitation measured at the same experimental station were 12 °C and 56 mm, respectively.

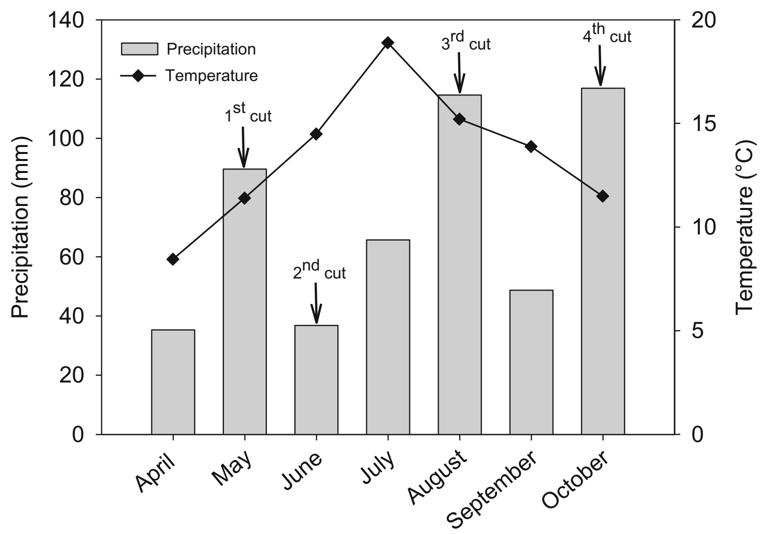

Fig. 1 Mean monthly air temperature and monthly precipitation during the experimental period from April to October 2014 measured at a climatic station near the experimental field

Experimental design and establishment of experimental plots

Sixteen seed mixtures composed of different combinations of red clover (*T. pratense* L., cultivar Rajah) perennial ryegrass (*L. perenne* L., cultivar Stefani) and three non-leguminous forbs: chicory (*C. intybus* L., cultivar Spadona), ribwort plantain (*P. lanceolata* L., wild type), and caraway (*C. carvi* L., cultivar Volhouden) were established in spring 2013 (Table 1). The species were sown in a replacement design—replacement of seeding proportion of one component in the mixture by other—based on the proportion of each species' seeding rate in a pure stand of 15, 4, and 12 kg ha^{-1} for ryegrass, red clover, and forbs, respectively, in 1.5 × 8-m plots in three replicates. Each mixture was treated with two levels of N fertiliser, 0 and 216 kg total N ha^{-1}, in the form of cattle slurry (containing 50% NH_4-N) applied in four split doses—91 kg at the start of the growing season in early April and the rest after the first, second, and third cuts in nearly equal amounts (39, 44, and 42 kg ha^{-1}, respectively). The plots were irrigated after the first and second cuts. The quantitative analysis of red clover BNF over a growing season was carried out in 2014 using the ^{15}N isotope dilution method as applied by Rasmussen et al. (2012). For this purpose, a subplot measuring 1 × 1 m was demarcated in each experimental plot and the soil was labelled by irrigating with ammonium sulphate 0.1 g N m^{-2} (atom% ^{15}N = 98) in early April 2014 to artificially enrich the soil ^{15}N above natural abundance.

Plant sampling and analysis

The shoot biomass was sampled by harvesting by hand to 5 cm stubble height in one 0.25 m^2 subplot per experimental plot four times during the growing season on 27 May, 30 June, 18 August, and 3 October. At each cut, unlabelled plant samples were collected adjacent to the experimental plots (but at least 5 m from the ^{15}N-labelled subplots). The biomass samples were sorted into individual species, dried at 80 °C for 24 h, and weighed. The dried samples were milled to a fine powder, packed in small tin capsules, and analysed for total N and atom% ^{15}N at the UC Davis Stable Isotope Facility, University of California, USA, on an ANCA-SL Elemental Analyser coupled to a 20–20 Mass Spectrometer using the Dumas dry-combustion method. The total N yield was quantified based on N concentrations and shoot DM yields of each species in the subplot.

Calculations

Biological N_2-fixation was quantified based on excess atom% ^{15}N in legume and non-legume species. Ryegrass and forbs grown in the same plot as red clover were used as non-legume reference plants, i.e. to estimate how much excess atom% ^{15}N in red clover was derived from soil. The percentage of red clover N derived from the atmosphere (%Ndfa) was calculated using the following equation (McNeill et al. 1994):

$$\%Ndfa = \left(1-\left(excess\ atom\%\ ^{15}\mathrm{N}\ legume/excess\ atom\%\ ^{15}\mathrm{N}\ reference\right)\right) \times 100$$

Table 1 Composition of the seed mixtures (percentage and amount are based on the seeding rate of each species in a pure stand)

Seed mixtures		Percentage of seed in the mixture (%)					Seeding rate (kg ha^{-1})				
		RC	GR	CH	PL	CA	RC	GR	CH	PL	CA
Pure stand	Red clover (RC)	100					4				
	Perennial ryegrass (GR)		100					15			
	Chicory (CH)			100					12		
	Ribwort plantain (PL)				100					12	
	Caraway (CA)					100					12
Two species	50RC + 50GR	50	50				2	7.5			
Three species	33CH + 33PL + 33CA			33	33	33			4	4	4
	20RC + 20GR + 60CH	20	20	60			0.8	3	7.2		
	40RC + 40GR + 20CH	40	40	20			1.6	6	2.4		
	20RC + 20GR + 60PL	20	20		60		0.8	3		7.2	
	40RC + 40GR + 20PL	40	40		20		1.6	6		2.4	
	20RC + 20GR + 60CA	20	20			60	0.8	3			7.2
	40RC + 40GR + 20CA	40	40			20	1.6	6			2.4
Five species	40RC + 40GR + 7CH + 7PL + 7CA	40	40	7	7	7	1.6	6	0.84	0.84	0.84
	20RC + 20GR + 20CH + 20PL + 20CA	20	20	20	20	20	0.8	3	2.4	2.4	2.4
	10RC + 10GR + 27CH + 27PL + 27CA	10	10	27	27	27	0.4	1.5	3.24	3.24	3.24

where excess atom% ^{15}N was calculated by subtracting natural ^{15}N abundance of the legume and reference (companion non-legume) species in unlabelled plots (background atom% ^{15}N) from the atom% ^{15}N of these species in ^{15}N-labelled plots. The background atom% ^{15}N values measured in both red clover and non-legume species were affected by neither cutting time nor plant species, so the average values measured in red clover of 0.3664 atom% and in ryegrass and forb species of 0.3678 atom% were used as background.

When calculating %Ndfa, the excess atom% ^{15}N of reference plants is assumed to reflect the ^{15}N signature of the soil N available for uptake by the legume (Carlsson and Huss-Danell 2014) and was therefore obtained from the non-legume species growing in the mixture with red clover. In mixtures containing more than one non-legume species, the average value for excess atom% ^{15}N of all non-legume species (forbs and ryegrass) was used as reference value. To obtain the reference value for calculating %Ndfa in pure stands of red clover, the average excess atom% ^{15}N value of all non-legume species grown in mixtures containing at least 40% red clover (according to seeding rate) or with higher red clover N proportions in the harvested biomass was used. This was based on the assumption that non-legumes growing in red clover-dominated mixtures more correctly reflect the ^{15}N signature of the soil N available to red clover than non-legumes growing as pure stands (Carlsson and Huss-Danell 2014). All excess atom% values of red clover and non-legumes are presented in Supplementary Table 1. The amount of BNF was calculated by multiplying %Ndfa with red clover N accumulation in shoots for each cut separately. The average %Ndfa for the whole growing season was estimated by dividing the total amount of BNF over the growing season by the total amount of red clover shoot N accumulated. The N uptake from soil was calculated subtracting total amount of BNF measured in shoots from the total shoot N accumulation.

Data analysis

The data were analysed in the open-source statistical program R (R Core Team 2016) (Version 3.1.0). Seasonal red clover N yield and N uptake and seasonal total DM and N yield data were log-transformed before analysis to obtain a normal distribution of residuals. Two-way analysis of variance was used to determine the effect of the two fixed factors (sown species composition and slurry application) on each of the dependent variables (DM yield, N yield, %Ndfa, amounts of BNF, and soil N uptake). The effect of cutting time on DM yield, N yield, %Ndfa, and amount of BNF for each slurry level was analysed using the linear mixed model, where sown species composition (fixed effect) and cutting time (repeated fixed effect) were independent variables with the blocks as a random variable and the plots were nested in the blocks. The model was tested using ANOVA. The pairwise comparisons were made by *lsmean* using the adjusted Tukey method. The probability of hypothesis rejection was tested at the 0.95 confidence level ($P < 0.05$).

Results

Weather condition during the experimental period

The weather conditions measured at the experimental site during the growing season (May to early October; Fig. 1) showed that the temperature was similar to the 30-year average, while the mean monthly precipitation was about 16% higher than the 30-year average at the same experimental station.

Dry matter production and botanical composition

The total DM production measured over the growing season was generally highest at the first and third cuts ($P < 0.001$) and lowest at the fourth cut and the difference was most pronounced without slurry application (data not shown). On a seasonal basis, red clover in pure stand had significantly higher DM yields than the other pure stands in plots without slurry application and significantly higher than ryegrass and caraway in plots with slurry application (Table 2). The three- and five-species mixtures containing forbs produced shoot DM yield up to 17 t ha^{-1}, but did not achieve significantly higher DM yields than the two-species red clover- ryegrass mixture, either with or without slurry application. On a seasonal basis, slurry application significantly increased total DM yield by 43 to 75% in pure stands of non-legumes and the three species mixture of forbs ($P < 0.001$). In the red clover-ryegrass-forb mixtures, the changes in yield ranged between −8 and 32%, with the two highest values in the five-species mixture containing 27% of each forb ($P < 0.001$) and the three-species mixture with 60% chicory in the seed mixture (Table 2).

The proportion of red clover in harvested biomass was often higher than the seeded proportion, with the percentage of total seasonal DM yield being 30 to 80% without and 30 to 60% with slurry application. Over the growing season, red clover produced the largest DM yield at the first and third cuts. Red clover had the highest proportions of total DM yield in the two-species mixture (Fig. 2). In the three-species mixtures, red clover generally had the highest proportion of total DM yield in the red clover-ryegrass-caraway combination followed by red clover-ryegrass-plantain mixtures. The red clover proportions often decreased in the red clover-ryegrass-chicory and five-species mixtures. In the three-species mixture of red clover, ryegrass, and chicory, the decrease in red clover DM yield, as compared to red clover-ryegrass mixture, was up to 57% without slurry and up to 40% with slurry application. The corresponding decrease in the red clover-ryegrass-plantain or caraway mixture was up to 36 and 27% without and with slurry application, respectively. Slurry application decreased the red clover proportion, especially in the two-species (red clover-ryegrass) and red clover-ryegrass-chicory mixtures (Fig. 2).

Ryegrass generally had higher DM yields in pure stand followed by the two-species mixture. In the red clover-ryegrass-forb mixtures, the ryegrass yielded more in the three species red clover-ryegrass-caraway mixture (Fig. 2). Slurry application increased ryegrass growth ($P < 0.001$) in the majority of the mixtures with the proportions of ryegrass generally increasing at the expense of red clover (Fig. 2).

Similar to ryegrass and red clover, the forb species also had the highest DM yields in pure stands. Chicory, in general, produced the highest yield in the mixture followed by plantain, whereas caraway generally produced relatively little DM (Fig. 2). Slurry application had a similar effect on the DM yield of chicory and plantain in the three species mixtures. In the five-species mixtures, it benefitted chicory DM yield at the expense of plantain. Biomass proportions of the species in the mixtures were more even in the fertilised plots, especially in the three-species red clover-ryegrass-plantain mixture.

N yield

Total seasonal N accumulation in different mixtures varied from 76 to 479 kg N ha^{-1} without and 126 to 491 kg N ha^{-1} with slurry application (Table 2). As the red clover dominated the mixtures, the differences in total N yields of the mixtures were mainly dependent on

Table 2 Total seasonal shoot dry matter (DM) and N yields measured at two levels of slurry application, 0 N and 216 kg total N ha^{-1} $year^{-1}$. Values are means (± SE; $n = 3$), with different letters within each column indicating a statistically significant ($P < 0.05$) difference between species compositions and "*" indicating a significant ($P < 0.05$) effect of slurry application within each variable

Seed mixtures		DM yield (t ha^{-1})		N yield (kg ha^{-1})	
		0 N	216 N	0 N	216 N
Pure stand	Red clover (RC)	15.4 ± 0.7^{e}	14.8 ± 0.9^{bc}	479 ± 7^{e}	491 ± 68^{d}
	Perennial ryegrass (GR)	4.5 ± 0.1^{a}	$7.9 \pm 0.8^{a*}$	76 ± 5^{a}	$127 \pm 12^{a*}$
	Chicory (CH)	7.1 ± 1.6^{ab}	$10.8 \pm 0.9^{ab*}$	106 ± 26^{ab}	$180 \pm 23^{a*}$
	Ribwort plantain (PL)	8.3 ± 0.9^{bc}	$12.1 \pm 1.0^{bc*}$	124 ± 15^{ab}	$190 \pm 16^{ab*}$
	Caraway (CA)	4.7 ± 0.3^{a}	$7.4 \pm 0.4^{a*}$	093 ± 6^{ab}	$134 \pm 8^{a*}$
Two species	50RC + 50GR	15.8 ± 0.9^{e}	14.5 ± 0.3^{bc}	468 ± 16^{e}	397 ± 28^{d}
Three species	33CH + 33PL + 33CA	8.9 ± 0.9^{bcd}	$12.8 \pm 0.7^{bc*}$	135 ± 10^{b}	$208 \pm 15^{abc*}$
	20RC + 20GR + 60CH	12.4 ± 1.4^{cde}	15.3 ± 1.0^{bc}	283 ± 34^{cd}	349 ± 36^{cd}
	40RC + 40GR + 20CH	15.2 ± 0.5^{e}	15.4 ± 0.6^{bc}	372 ± 29^{cde}	359 ± 28^{d}
	20RC + 20GR + 60PL	15.6 ± 1.4^{e}	15.9 ± 1.0^{bc}	392 ± 52^{cde}	411 ± 28^{d}
	40RC + 40GR + 20PL	15.8 ± 1.0^{e}	16.7 ± 0.1^{c}	416 ± 26^{de}	418 ± 33^{d}
	20RC + 20GR + 60CA	14.5 ± 1.7^{e}	14.4 ± 1.8^{bc}	389 ± 53^{cde}	349 ± 52^{cd}
	40RC + 40GR + 20CA	14.8 ± 0.6^{e}	15.5 ± 0.9^{bc}	426 ± 9^{de}	403 ± 22^{d}
Five species	40RC + 40GR + 7CH + 7PL + 7CA	13.7 ± 0.1^{e}	16.5 ± 1.2^{c}	337 ± 1^{cde}	402 ± 39^{d}
	20RC + 20GR + 20CH + 20PL + 20CA	13.4 ± 1.5^{de}	15.0 ± 0.1^{bc}	324 ± 54^{cde}	331 ± 14^{cd}
	10RC + 10GR + 27CH + 27PL + 27CA	11.6 ± 0.9^{cde}	$15.3 \pm 0.4^{bc*}$	243 ± 29^{c}	$331 \pm 26^{bcd*}$

the red clover proportion. On a seasonal basis, red clover in pure stands produced the highest N yields followed by the two-species mixture without slurry and the three-species red clover-ryegrass-plantain or caraway mixtures with a high seeding proportion of red clover with slurry application. The total N yields did not differ significantly between the mixtures containing red clover with slurry application. Without slurry application, the N yield in the red clover-ryegrass-chicory and a five-species mixture with the highest seeding proportion of forbs was significantly lower ($P < 0.001$) than in the two-species mixture. Slurry application increased

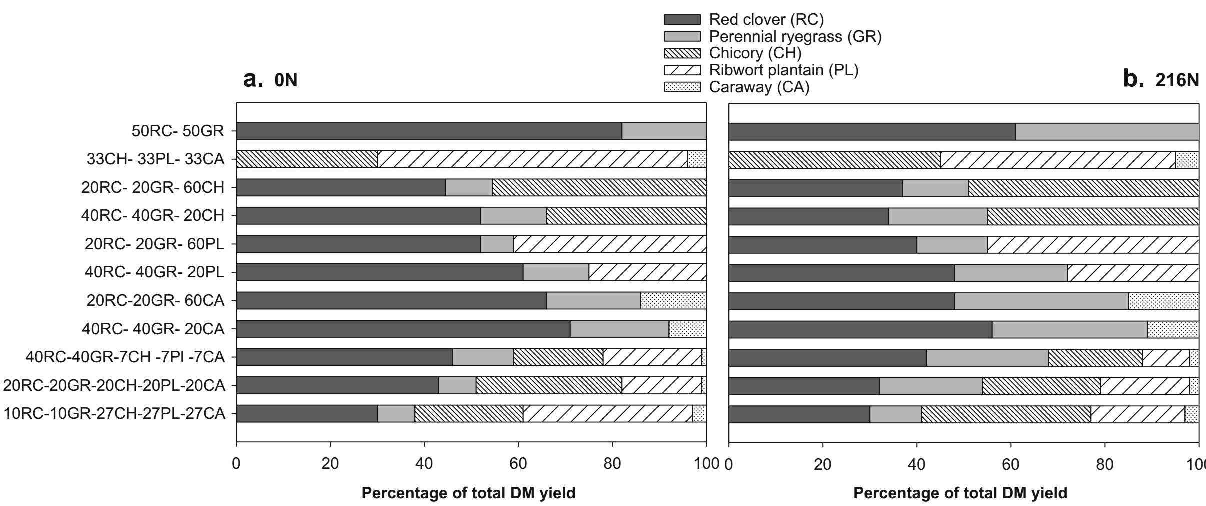

Fig. 2 Botanical composition of above-ground dry matter (DM) yield of different species mixtures grown without (0 N) and with (216 kg total N ha^{-1}) slurry application ($n = 3$) sampled four times over the growing season in 2014

($P < 0.001$) the seasonal total N yield for pure stands of non-legumes and the three-species mixture of forbs by 44 to 70%. The changes in N yield of red clover-ryegrass-forb mixtures ranged between −5 and 36%, with a greater effect in the five species mixture with a high seeding proportion of forbs.

Percentage and amount of red clover N derived from BNF

The atom% ^{15}N measured in ^{15}N-labelled plots in all five species at all four sampling occasions was sufficiently above the natural ^{15}N abundance and there was a clear distinction between red clover and non-legumes to calculate the %Ndfa. The excess atom% ^{15}N was highest at the first cut and decreased for succeeding cuts. In mixtures, the ryegrass was often more enriched compared to forbs (Supplementary Table 1).

The percentage of N in red clover derived from BNF significantly increased ($P < 0.001$) in mixtures compared to the pure stands of red clover (Table 3). The seasonal %Ndfa in red clover grown in mixtures was consistently above 90%, and there was no significant difference between mixture compositions both with and without slurry application (Table 3). The percentages of red clover BNF were consistently high during the first three cuts and lower at the fourth cut ($P < 0.001$) (Supplementary Table 2).

Slurry applications of 216 kg total N ha^{-1} generally did not significantly lower %Ndfa in the mixtures, although there were individual exceptions (Supplementary Table 2). The effect on the specific mixture varied depending on cutting time and composition of seed mixtures with a significant interactive effect ($P < 0.001$) for both levels of slurry application. On a seasonal basis, the slurry application significantly ($P < 0.001$) decreased %Ndfa in the pure stand of red clover and in the red clover-ryegrass-chicory mixture with a high seeding proportion of red clover (Table 3).

The amount of BNF differed depending on seed mixture and cut, with significant interactive effects, especially with slurry application ($P < 0.05$). The amount of BNF was closely related to the pattern of red clover DM and N accumulation. There was a strong linear association between the amount of N_2 fixed and both total N yield ($R^2 = 0.81$ with slurry and 0.94 without slurry and $P < 0.001$) and red clover N yield ($R^2 =$ 0.97 both with and without slurry and $P < 0.001$). The total amount of seasonal BNF in different mixtures ranged between 105 and 390 kg N ha^{-1} without and from 130 to 340 kg N ha^{-1} with slurry application (Table 3). The amount of BNF was highest in the pure stand of red clover followed by the two-species red clover-ryegrass mixture. Red clover in the three-species mixtures with ryegrass and plantain or caraway fixed in excess of 200 kg N ha^{-1} year^{-1} at both levels of slurry application, which in most cases was not significantly different from red clover in the pure stand and the two-species red clover-ryegrass mixture. The amount of BNF was suppressed by up to 70% without and 50% with slurry application compared to red clover-ryegrass mixture in the red clover-ryegrass-chicory and five-species mixtures, especially with a high seeding proportions of forbs, compared to other species compositions (Table 3), which was mainly caused by a decrease in the proportion of red clover in the harvested biomass (Fig. 2).

Slurry application generally lowered the amount of BNF in the majority of the mixtures, which was most pronounced at the first and second cuts (data not shown). The amount of BNF declined mainly due to a decrease in the red clover biomass proportion in treatments with slurry application (Fig. 2). On an annual basis, slurry application lowered the seasonal BNF by up to 35%, with a larger effect in the two- and three-species mixtures than in the five-species mixtures. In three-species mixtures, there was a tendency for the effect of slurry application to be larger with a high seeding proportion of red clover. The effect of slurry application was, however, significant only in the two-species mixture ($P < 0.001$).

N uptake in red clover and non-legumes

The uptake of soil N in red clover was highest in the pure stand followed by the two-species mixture of red clover and ryegrass (Table 4). The range of difference due to slurry application was small across all the treatments and was not statistically significant for red clover and all the forb species in the mixtures.

When included in unfertilised red clover-ryegrass-forb mixtures with a 20% or lower proportion in the seed mixture, red clover took up significantly less soil N than in the two-species mixture with ryegrass (Table 4). While all non-legume pure stands significantly increased their N uptake in response to slurry application (Table 2), only ryegrass (i.e. none of the forbs) significantly increased its N uptake with slurry application

Table 3 Total seasonal red clover N yields and percentage (%Ndfa) and amount of red clover N derived from the atmosphere measured in shoots under two levels of slurry application, 0 and 216 kg total N ha^{-1} year^{-1}. Values are means (± SE; $n = 3$), with different letters within each column indicating statistically significant ($P < 0.05$) differences between species compositions and "*" indicating a significant ($P < 0.05$) effect of slurry application within each variable

Seed mixtures		Red clover N yield (kg ha^{-1})		%Ndfa		N_2-fixation (kg ha^{-1})	
		0 N	216 N	0 N	216 N	0 N	216 N
Pure stand	Red clover	479 ± 7^{d}	491 ± 68^{b}	81.2 ± 4.6^{a}	69.3 ± 2.8^{a}*	389 ± 21^{e}	339 ± 42^{b}
Two species	50RC + 50GR	412 ± 14^{cd}	283 ± 40^{ab}*	92.9 ± 1.3^{b}	91.4 ± 2.1^{b}	383 ± 18de	258 ± 36^{ab}*
Three species	20RC + 20GR + 60CH	164 ± 33^{ab}	170 ± 44^{a}	96.8 ± 0.7^{b}	94.0 ± 0.8^{b}	159 ± 33^{ab}	159 ± 39^{a}
	40RC + 40GR + 20CH	240 ± 34^{bcd}	167 ± 35^{a}	95.6 ± 1.0^{b}	89.8 ± 2.8^{b}*	229 ± 32^{abcd}	148 ± 26^{a}
	20RC + 20GR + 60PL	253 ± 49^{bcd}	230 ± 24^{ab}	96.9 ± 0.6^{b}	94.4 ± 0.7^{b}	245 ± 46^{abcde}	217 ± 22^{ab}
	40RC + 40GR + 20PL	297 ± 33^{bcd}	247 ± 48^{ab}	95.1 ± 0.5^{b}	92.3 ± 1.9^{b}	283 ± 32^{bcde}	226 ± 41^{ab}
	20RC + 20GR + 60CA	298 ± 57^{bcd}	210 ± 47^{a}	97.3 ± 0.3^{b}	95.5 ± 0.2^{b}	290 ± 55^{bcde}	200 ± 45^{ab}
	40RC + 40GR + 20CA	333 ± 10^{bcd}	265 ± 13^{ab}	94.7 ± 1.0^{b}	92.7 ± 0.8^{b}	315 ± 7^{cde}	245 ± 10^{ab}
Five species	40RC + 40GR + 7CH + 7PL + 7CA	197 ± 13^{abc}	207 ± 33^{a}	94.9 ± 0.9^{b}	91.3 ± 1.2^{b}	187 ± 13^{abc}	189 ± 28^{ab}
	20RC + 20GR + 20CH + 20PL + 20CA	183 ± 53^{ab}	139 ± 23^{a}	95.0 ± 0.6^{b}	94.7 ± 0.2^{b}	174 ± 51^{abc}	132 ± 22^{a}
	10RC + 10GR + 27CH + 27PL + 27CA	108 ± 32^{a}	138 ± 28^{a}	96.9 ± 0.4^{b}	95.0 ± 0.1^{b}	105 ± 30^{a}	131 ± 27^{a}

RC: red clover, GR: perennial ryegrass, CH: chicory, PL: ribwort plantain, CA: caraway

when grown in red clover-ryegrass-forb mixtures. Chicory and plantain in red clover-ryegrass-forb mixtures almost always took up more N than perennial ryegrass—the only exception was the fertilised five-species mixtures with 40% of ryegrass (Table 4). Sum of non-legume N uptake increased by up to 150 and 80% without and with slurry application, respectively, in red clover-ryegrass-forb mixtures as compared to red clover-ryegrass mixture, which was more pronounced in the three-species mixture containing red clover, ryegrass, and plantain or caraway and in five species mixtures.

Discussion

Our focus was on investigating how new non-legume species (forbs) with different functional traits influence sward production, legume growth, and BNF when included in the traditional red clover-ryegrass mixture.

Red clover growth dynamics and sward competition

We found a yield advantage in mixtures containing red clover compared to non-legume pure stands and the three-species mixture of forbs. On the other hand, in the presence of red clover, the total DM and N yield were generally not affected by plant species diversity, seeding proportion of red clover, or slurry treatment, neither in the present experimental year nor in the following year in the same field experiment as reported by Cong et al. (2017). The second experimental year showed lower total DM yield and a higher ryegrass proportion in expense of red clover, whereas the proportion of forbs remained similar in both years (Cong et al. 2017). The total DM and N yield of the mixtures were higher or comparable to similar two-species mixtures of grass and clover (Rasmussen et al. 2012) and multi-species forage legume, grass, and non-legume forb mixture (Pirhofer-Walzl et al. 2012) previously measured at the same location or grassland production at other regions in the Europe (Oberson et al. 2013; Pirhofer-Walzl et al. 2013; Anglade et al. 2015). This demonstrated high herbage production potential of the sward containing forbs in the present low-input system.

Red clover generally dominated in the mixtures regardless of species composition and seeding proportions of red clover and non-legumes. Thus, red clover defined the DM and N yield of the mixtures, especially without slurry application. This reflects the strong competitive ability of red clover for above- and below-ground resources (Rasmussen et al. 2012) and the competitive advantage of N_2-fixing species from BNF over non-fixing species to sustain N nutrition under no N

Table 4 Seasonal N uptake from soil pools including slurry N in red clover, perennial ryegrass, chicory, ribwort plantain, and caraway measured under two levels of slurry application, 0 and 216 kg total N ha^{-1} $year^{-1}$. Values are means (± SE; $n = 3$), with different letters within each column indicating statistically significant ($P < 0.05$) differences between species compositions and "*" indicating a significant ($P < 0.05$) effect of slurry application within each variable

Seed mixtures		N uptake (kg ha^{-1})									
		Red clover		Perennial ryegrass		Chicory		Ribwort plantain		Caraway	
		0 N	216 N	0 N	216 N	0 N	216 N	0 N	216 N	0 N	216 N
Pure stand	Red clover	90 ± 22^{e}	152 ± 30^{c}								
Two species	50RC + 50GR	29 ± 4^{d}	25 ± 8^{b}	56 ± 3^{bc}	114 ± 13^{e}*						
Three species	33CH + 33PL + 33CA					45 ± 25^{a}	98 ± 27^{ab}	83 ± 15^{ab}	98 ± 2^{bc}	8 ± 1^{ab}	12 ± 2^{a}
	20RC + 20GR + 60CH	5 ± 1^{ab}	11 ± 4^{ab}	22 ± 6^{ab}	40 ± 11^{ab}	98 ± 9^{a}	138 ± 21^{ab}				
	40RC + 40GR + 20CH	11 ± 3^{abcd}	19 ± 9^{ab}	39 ± 2^{abc}	63 ± 14^{abcd}*	93 ± 12^{a}	130 ± 9^{ab}				
	20RC + 20GR + 60PL	8 ± 3^{abc}	13 ± 3^{ab}	21 ± 2^{ab}	46 ± 9^{abc}*			119 ± 6^{b}	135 ± 7^{c}		
	40RC + 40GR + 20PL	14 ± 1^{bcd}	20 ± 8^{ab}	45 ± 5^{abc}	75 ± 17^{bcd}*			73 ± 10^{ab}	97 ± 4^{bc}		
	20RC + 20GR + 60CA	8 ± 2^{abc}	10 ± 3^{ab}	43 ± 5^{abc}	88 ± 3^{de}*					48 ± 3^{c}	51 ± 6^{b}
	40RC + 40GR + 20CA	18 ± 4^{cd}	19 ± 3^{ab}	63 ± 10^{c}	93 ± 5^{de}*					30 ± 11^{bc}	45 ± 10^{b}*
Five species	40RC + 40GR + 7CH + 7PL + 7CA	10 ± 1^{abcd}	19 ± 5^{ab}	34 ± 5^{abc}	80 ± 11^{cde}	46 ± 23^{a}	68 ± 20^{a}	56 ± 22^{a}	36 ± 4^{a}	4 ± 2^{a}	10 ± 6^{a}
	20RC + 20GR + 20CH + 20PL + 20CA	9 ± 2^{abc}	7 ± 1^{a}	18 ± 5^{a}	59 ± 5^{abcd}*	77 ± 1^{a}	74 ± 20^{a}	42 ± 1^{a}	53 ± 4^{ab}	5 ± 1^{a}	7 ± 1^{a}
	10RC + 10GR + 27CH + 27PL + 27CA	4 ± 1^{a}	7 ± 1^{a}	14 ± 5^{a}	32 ± 6^{a}	48 ± 9^{a}	97 ± 13^{ab}	66 ± 8^{a}	52 ± 2^{ab}	7 ± 2^{ab}	12 ± 2^{a}

RC: red clover, GR: perennial ryegrass, CH: chicory, PL: ribwort plantain, CA: caraway fertilisation (Carlsson and Huss-Danell 2003). Hence, the present study indicates that a potential yield advantage expected from increasing the number of species may be overshadowed by the high productivity of a competitive forage legume—in this case red clover.

The red clover proportions of total DM and N yield were suppressed in red clover-ryegrass-chicory and five-species mixtures, but red clover showed a competitive advantage when grown with plantain and caraway in three-species mixtures. Our results showed that chicory competed strongly with the other species in herbage production, which is in line with the observations of Goh and Bruce (2005) and may be explained by the plant functional traits. The tall rosette plant and broad prostrate leaves of chicory would have shaded neighbouring plants (Søegaard et al. 2013), and its deep-growing roots may compete successfully for nutrients and water (Pirhofer-Walzl et al. 2013; Thorup-Kristensen 2006). Despite better competitiveness in the mixture, the plantain may have favoured the growth of red clover by its more upright leaves letting in more light (Søegaard et al. 2013). We observed increased growth of caraway later in the growing season, which could be related to its initial energy investment for establishing a large root system (Hakala et al. 2008; Søegaard et al. 2013).

Comparing ryegrass and red clover, the main finding was that addition of slurry largely increased the proportion of ryegrass at the expense of red clover in the sward. Thus, although red clover generally dominated the swards, its growth varied with non-legume seeding proportions and slurry application. The large span of red clover proportion (from 30 to 80%) of the sward shoot biomass did however not influence total herbage DM yields of the mixtures (Table 2), indicating that complementary resource acquisition by the included species compensated for the variation in red clover DM yield. On the other hand, without slurry application, total N yields were lower in mixtures with a lower proportion of red clover in the harvested shoot biomass (Table 2). This highlights the role of legumes for sustaining high N yields in unfertilised grasslands and shows that the inclusion of competitive forbs such as chicory may have negative effects on the total N yield in grasslands by suppressing legume growth and N_2-fixation.

Proportion of red clover N derived from BNF (%Ndfa)

The proportion of N in red clover derived from BNF (%Ndfa) was stimulated in all the mixtures compared to the pure stand of red clover, but it was remarkably consistent across the two-, three-, and five-species mixtures, both with and without slurry application. Hence, we could not confirm our first hypothesis that red clover reliance on BNF would increase with increasing species diversity of companion non-legumes. Previous studies have reported that the %Ndfa is primarily influenced by legume production, soil N availability, and competition for the available soil N among co-existing non-legumes (e.g. Ledgard and Steele 1992; Høgh-Jensen and Schjoerring 1997; Carlsson and Huss-Danell 2003; Nyfeler et al. 2011). Carlsson et al. (2009), Nyfeler et al. (2011), and Oberson et al. (2013) have shown a stimulatory effect of higher proportions of N sinks (non-legumes) on %Ndfa. In contrast to those studies, we found no relation between non-legume proportion (ranging from 20 to 70%), non-legume N uptake, species diversity, and composition on %Ndfa in red clover for the two-, three-, and five-species mixtures. Furthermore, we found no effect of slurry application on %Ndfa in the majority of the mixtures, even in the mixtures dominated by red clover (i.e. a significant effect of slurry application on %Ndfa was only detected in the red clover pure stand). This fails to support our second hypothesis and contrast with the previous findings that demonstrated negative effects of slurry application on %Ndfa in red clover (e.g. Pirhofer-Walzl et al. 2012; Rasmussen et al. 2012), also when grown in mixtures with non-legumes. Carlsson et al. (2009) found that increasing soil N levels affect %Ndfa, depending on companion non-legume species' competition for available soil N. They found %Ndfa to decrease with increases in soil N level in species-poor communities and to increase in species-rich communities containing grass. However, we found no such response to slurry application when comparing the proportion of different functional groups in the different mixtures included in the present study.

The lack of an effect of slurry on %Ndfa when red clover was growing in a mixture with ryegrass and forbs could be due to very low soil N levels. If the soil N status was initially very low, then application of fertiliser N would benefit the non-legumes of the sward, which was indeed the case in our experiment. However, in most mixtures, it was only the proportion of ryegrass in harvested DM which increased with slurry application, whereas the proportion of forbs in most cases did not increase. Also, the increased soil N uptake in response to slurry application was significant only for ryegrass in the mixtures, not for forbs. These observations point to the likely explanation that soil N availability was limiting only for ryegrass and that the forbs could acquire sufficient amounts of N—potentially by uptake from deeper soil layers—for their growth even without slurry application. This is in line with a parallel study in the same experimental set up on N transfer from red clover to the ryegrass and forbs, showing that forbs to a greater extent relied on soil N compared to ryegrass; the latter in turn relying on red clover-derived N (Dhamala et al. 2017a). The observed increases in the proportion of harvested DM and N uptake by ryegrass in slurry-fertilised mixtures support the hypothesis that ryegrass efficiently competes for available soil N, thereby stimulating high %Ndfa in companion legumes even if the mixture is fertilised with N (e.g. Carlsson and Huss-Danell 2003; Palmborg et al. 2005; Carlsson et al. 2009). In addition to the complementarity between soil N uptake and BNF, we suggest that the ryegrass is also a strong competitor for N derived from red clover via rhizodeposition and N transfer (Dhamala et al. 2017a). Consequently, when growing in mixtures with ryegrass, red clover would not be able to re-assimilate its deposited N (root exudates), which could have provoked the red clover to rely on its own BNF. This explanation is supported also by the finding of Dhamala et al. (2017b), where legume %Ndfa was not affected in the mixed stands containing forage legumes only, which was likely due to the absence of non-legume competition for legume access to their N exudates. Hence, ryegrass competition with red clover for red clover-derived N via rhizodeposition or N transfer offers an alternative explanation for the observed pattern of high %Ndfa in all treatments. This explanation is also in line with findings by Carlsson et al. (2009), suggesting that species composition and functional traits (e.g. efficient soil N uptake by competitive grasses) are more important than species richness per se for an effect of companion non-legumes on legume BNF.

Studies have suggested that %Ndfa estimated using ^{15}N isotope dilution method might be confounded by uneven spatiotemporal distribution of ^{15}N-labelled fertiliser in the soil profile (e.g. Unkovich et al. 2008; Burchill et al. 2014). Such unevenness in the soil ^{15}N enrichment might lead to different N uptake patters of the legumes and reference plants and violate the assumption that ^{15}N enrichment of the reference plants represents the ^{15}N enrichment of legume N derived from the soil. To minimise the risk of confounding effects of spatiotemporal variations in soil ^{15}N enrichment and contrasting N uptake patterns between the legume and different non-legume reference plants, the average excess atom% ^{15}N of all non-legumes growing in mixtures with red clover were used as the reference ^{15}N value in the calculation of %Ndfa, as suggested by (e.g. Jacot et al. 2000; Unkovich et al. 2008; Carlsson and Huss-Danell 2014). Jørgensen et al. (1999) suggested that identical N uptake pattern between N_2-fixing and non-fixing reference plants plays more important role than the temporal variation in the soil ^{15}N enrichment for reliable estimation of %Ndfa. In the present study, we observed a similar ^{15}N enrichment pattern among the different species at each cut, i.e. while the overall ^{15}N enrichment decreased over time the differences between species remained similar (Supplementary Table 1). Furthermore, this study aimed at evaluating the effect of different species composition (including forbs in the ryegrass-red clover mixture) on %Ndfa at each cutting time or on the seasonal basis. Hence, we do not emphasise the temporal variations between cuts.

The amount of red clover N derived from BNF

Since we observed a consistently high %Ndfa across two-, three-, and five-species mixtures, the amount of BNF in mixtures generally followed the same trend as the DM and N yield of the red clover. This confirms that legume DM production is the main factor controlling the amount of N derived from BNF, as shown in several previous studies (e.g. Carlsson and Huss-Danell 2003; Dahlin and Stenberg 2010a; Unkovich et al. 2010; Anglade et al. 2015). Red clover in three species mixtures with plantain or caraway fixed in excess of 200 kg N ha^{-1}, which was comparable to red clover in a pure stand and the two-species red clover-ryegrass mixture. The amount of BNF was only affected in the mixture with a high chicory content and with slurry application, which reduced the proportion of red clover in the sward. This showed that selection of forb species and their seeding proportions are important in the multi-species forb-based swards for a balance between legume and non-legume biomass proportions.

The amount of shoot N derived from red clover BNF (105–400 kg N ha^{-1} $year^{-1}$) in the present experiment was lower than the highest amount recorded (545 kg N ha^{-1} $year^{-1}$) in red clover in Europe (Anglade et al. 2015). However, it was within the previously reported range in legumes of European grasslands (100–380 kg N ha^{-1} year^{-1}) (Lüscher et al. 2014) and comparable to the reported amounts of BNF in red clover in northern European grasslands (373 kg N ha^{-1} year^{-1}, Carlsson and Huss-Danell 2003; 324 with and 357 kg ha^{-1} year^{-1} without slurry, Rasmussen et al. 2012). Thus, the present organic temporary grassland system obtained large amounts of N from red clover BNF and forb species and seeding proportion in the mixture were the main influences on the amount of BNF via their effect on the share of legumes in the harvested biomass.

Conclusions

Our study did not show an effect on red clover N proportion from biological N_2-fixation when including forbs in red clover-ryegrass mixtures. Neither did we observe a negative effect of cattle slurry on the red clover proportion of N derived from biological N_2-fixation when in mixture, indicating a strong competition from non-legumes for available N sources in soil. The red clover yield in the harvested biomass defined, as previously reported, the amount of N originating from biological N_2-fixation, with some variation due to the seeding proportions of the species, competitive ability of the forbs, and application of slurry.

We conclude that forbs can be included in temporary multi-species grasslands without negative effects on herbage yield, total N yield, and amount of biological N_2-fixation, provided that the mixtures do not include very high proportions of chicory in the seed mixture to retain a balance between legume and non-legume biomass proportions.

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